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Precise T cell recognition programs designed by transcriptionally linking multiple receptors

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Abstract

Living cells often identify their correct partner or target cells by integrating information from multiple receptors, achieving levels of recognition that are difficult to obtain with individual molecular interactions. In this study, we engineered a diverse library of multireceptor cell-cell recognition circuits by using synthetic Notch receptors to transcriptionally interconnect multiple molecular recognition events. These synthetic circuits allow engineered T cells to integrate extra-

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Data and materials availability: All data are available in the main text or the supplementary materials. Expression plasmids are available from Addgene under a material transfer agreement. Cell lines, other than primary T cells, are available upon request.

Competing interests: W.A.L., K.T.R., J.Z.W., G.M.A., and D.S. are inventors on patents related to this work. W.A.L., K.T.R., and J.Z.W. receive licensing fees for patents that were licensed by Cell Design Labs and are now part of Gilead. W.A.L. is on the scientific advisory board of Allogene Therapeutics. K.T.R. is a cofounder of Arsenal Biosciences. J.Z.W. is currently an employee of Arsenal Biosciences. A.R. has received honoraria from consulting with Amgen, Bristol-Myers Squibb, Chugai, Genentech, Merck, Novartis, Roche, and Sanofi; is or has been a member of the scientific advisory board and holds stock in Advaxis, Apricity, Arcus Biosciences, Bioncotech Therapeutics, Compugen, CytomX, Five Prime, FLX-Bio, ImaginAb, Isoplexis, Kite-Gilead, Lutris Pharma, Merus, PACT Pharma, Rgenix, and Tango Therapeutics; and has received research funding from Agilent and from Bristol-Myers Squibb through Stand Up to Cancer (SU2C).

SUPPLEMENTARY MATERIALS

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and intracellular antigen recognition, are robust to heterogeneity, and achieve precise recognition by integrating up to three different antigens with positive or negative logic. A three-antigen AND gate composed of three sequentially linked receptors shows selectivity in vivo, clearing three-antigen tumors while ignoring related two-antigen tumors. Daisy-chaining multiple molecular recognition events together in synthetic circuits provides a powerful way to engineer cellular-level recognition.

Precise cell-cell recognition is critical throughout biology. Developing cells, neurons, and immune cells must identify their correct partner or target cells with extraordinary specificity. Such precise cellular recognition is mediated by multiple surface receptors that function in an integrated, combinatorial manner (1). Precise cellular recognition is also a central goal in treating diseases such as cancer, but current therapeutic approaches [antibodies, bispecific antibodies, or chimeric antigen receptors (CARs)] primarily rely on identifying malignant cells by interaction with a single cancer-associated antigen (Fig. 1A, left). These approaches, although powerful in some cases, are limited, because most cancer cells lack a single antigen whose expression cleanly distinguishes them from healthy cells (2). CAR T cells—which are engineered to redirect cytotoxic activity against a targeted extracellular antigen—have caused toxic on-target off-tumor cross-reactions with normal tissues in clinical trials (3-8), because many potential CAR tumor antigen targets in solid cancers are also found in normal epithelial tissues. Bioinformatic analyses, however, indicate that combinatorial antigen pattern recognition could enable more-specific cancer targeting (9-11). Thus, we aim to design tailored recognition programs that exploit discriminatory combinatorial features of target disease cells.

Engineered cell therapies are an opportune platform for constructing combinatorial recognition programs, because, in principle, multiple molecular recognition systems could be assembled into higher-order cellular recognition programs through coordinated intracellular regulation (Fig. 1A, right). We previously designed synthetic Notch (synNotch) receptors that link an antigen recognition domain [e.g., a single-chain antibody (scFv)] to a transcriptional regulatory domain, which, only upon target antigen binding, induces expression of a user-defined, genetically encoded payload (12). By using a synNotch receptor to drive expression of a CAR, directed against a second antigen, we engineered a two-antigen AND gate in which the circuit leads to T cell activation and target cell killing only when the target cell expresses both the synNotch and CAR antigens on its surface (13). This AND gate, however, represents only a small fraction of the possible combinatorial recognition circuits that could be engineered. In this study, we used multiple synNotch receptors as flexible regulatory connectors to daisy-chain, or link together, a broader range of receptors and outputs into circuits that can achieve a more complete set of cell-cell recognition behaviors (Fig. 1A).

One simple but impactful recognition program would integrate extracellular and intracellular recognition (Fig. 1B). CAR T cells are generally restricted to recognizing extracellular antigens, which limits their ability to read and harness the ~75% of the proteome that is intracellular (including many cancer antigens) (14). We designed such an external-internal recognition circuit to recognize melanoma cells. The intracellular melanocyte antigen

MART1 is presented on the cell surface by major histocompatibility complexes (MHCs). A T cell receptor (TCR) directed against MART1 showed antimelanoma effects in clinical trials, but anti-MART1 T cells also induced severe rash or vitiligo, uveitis, and hearing loss due to on-target off-tumor attack of normal melanocytes that also express MART1 (Fig. 1C) (6). RNA sequencing data from 53 human melanoma cell lines (15, 16) shows that many patient-derived melanoma lines also overexpress the surface antigen MET (hepatocyte growth factor receptor) (~50% of samples with transcripts per million > 5; fig. S1, A and B), which is only weakly expressed in normal adult melanocytes (17-19). Thus, a recognition circuit that requires MET and MART1 could be used to distinguish between melanoma cells and normal melanocytes. We constructed an anti-MET synNotch→anti-MART1 [human leukocyte antigen A2 (HLA-A2)] TCR circuit (fig. S1, C and D) and showed that, unlike standard constitutive anti-MART1 T cells, CD8⁺ (killer) T cells expressing this circuit did not show T cell activation, cytokine production, proliferation, or killing directed against normal adult melanocytes (Fig. 1D and fig. S1, E and F). In contrast, when challenged with MET⁺ (M202) patient-derived melanoma cell lines, the MET→MART1 TCR T cells showed strong proliferation and cytotoxicity against these MET⁺/MART1⁺ tumor cells (Fig. 1E). MET⁻ (M262) melanoma cell lines, however, did not activate these T cells. MART1 TCR T cells and MET→MART1 TCR T cells had similar cytotoxic capability against dual-antigen-positive targets, demonstrating that the circuit's increased precision does not sacrifice efficacy (Fig. 1E). Thus, synNotch→TCR circuits can function robustly, and they offer a general solution for improving the specificity of therapeutic TCRs [such as MART1 or MAGEA3 (melanoma-associated antigen 3)] (fig. S1, G, to I) with toxic cross-reactivities.

scFvs raised against specific peptide-MHC (pMHC) complexes have been used to engineer CARs that detect MHC-presented intracellular antigens (20). We used these anti-pMHC scFvs as recognition heads for synNotch receptors (referred to as “inNotch” receptors) (fig. S2A). As an example, we validated that an inNotch receptor with an HLA-A2/AFP (alpha-fetoprotein) recognition domain (20) drives induction of a blue fluorescent protein (BFP) reporter in an antigen-dependent manner (fig. S2B). We tested whether the anti-HLA-A2/AFP inNotch receptor could be incorporated into AND gates regulating either CAR or TCR expression. T cells engineered with the anti-AFP inNotch controlling either an anti-HER2 (human epidermal growth factor receptor 2) CAR (internal→external circuit) or anti-HLA-A2/NY-ESO1 (cancer/testis antigen 1) TCR (internal→internal circuit) acted as AND gates and specifically killed the intended dual-positive target cells (fig. S2C, to E).

A second broad and general class of combinatorial recognition strategy involves the incorporation of negative antigen inputs. Recent bioinformatic analysis indicates that negative information (i.e., NOT gate antigens expressed in normal tissues but not in the target cancer cells) could be powerful in preventing toxic cross-reactions (11). Earlier studies have explored the design of an inhibitory-CAR receptor (iCAR) (21), which fuses NOT antigen recognition domains with intracellular signaling domains of immune inhibitory receptors to override CAR killing. However, these iCAR receptors require very high levels of expression of both the iCAR and the NOT antigen and have not proven to be highly robust. Thus, it appears that directly overriding CAR activity at the membrane-proximal signaling level is challenging (21).

An alternative strategy for engineering a NOT gate antigen input might be to override T cell function downstream from CAR signaling. For example, potent CAR T cell function requires both T cell activation and proliferation. Thus, an effective NOT gate strategy could be to direct negative inputs to induce apoptosis, thereby blocking critical T cell expansion. We therefore constructed a synNotch circuit that activated expression of the proapoptotic factor tBID (truncated BH3-interacting domain death agonist) (fig. S3A). This circuit, which we refer to as OFF-Notch, rapidly induced T cell death when stimulated (fig. S3, B to E). To incorporate this OFF-Notch receptor as a NOT gate in a three-antigen circuit that also includes an AND gate requires two orthogonal synNotch platforms with distinct transcriptional regulatory domains. We found that GAL4-VP64 and LEXA-VP64 synNotch receptors could yield robust independent responses with no cross-talk when coexpressed in primary human T cells (fig. S4).

With these components in hand, we combined an anti-HER2 NOT gate with an AND gate [recognizing the model antigens GFP (green fluorescent protein, surface-expressed) and CD19] (Fig. 2A). The composite circuit showed the predicted target selectivity—when CD8⁺ T cells expressing the circuit were mixed with K562 tumor cells engineered to express different combinations of the antigens HER2, surface GFP, and CD19 (fig. S5), all tumor cells expressing HER2 (NOT antigen) were spared (Fig. 2B and fig. S6B). In contrast, only those target cells expressing the on-target antigen combination of surface GFP, CD19, and NOT HER2 were killed. Selectivity was maintained at the level of T cell proliferation—GFP⁺/CD19⁺ tumor cells induced potent T cell expansion (3.7 ± 0.2 divisions), but this proliferation was significantly blocked by target cells that also expressed HER2 (in addition to GFP⁺/CD19⁺) (1.9 ± 0.1 divisions, $P = 0.0002$) (Fig. 2C). Thus, we were able to build a negative regulatory circuit that counteracts CAR activity at a downstream level (i.e., cell survival), effectively blocking both killing and proliferation responses that play a key role in on-target off-tumor toxicity. For clinical application of this circuit, local induction of cell death must be carefully balanced with sufficient expansion in targeted tumor and CAR T cell efficacy, and it may require induction of alternative payloads that have a smaller effect on T cell survival (fig. S3E).

Interestingly, this NOT gate worked best when counteracting a positive AND gate circuit (synNotch→CAR) rather than when counteracting a constitutively expressed CAR (fig. S6A). The delay required for antigen-induced tBID expression (fig. S3C, and D) is likely too long to effectively counteract the immediate signaling of a constitutively expressed CAR. Placing CAR expression under analogous synNotch control likely works better because the positive and negative branches of the circuit are dynamically well matched—both the CAR and tBID are induced at similar time scales, allowing more even competition between the pathways. Thus, matching kinetics is likely a critical design principle for circuits incorporating both positive and negative regulation.

Multiantigen AND gates—circuits that require three or more antigens to trigger T cell recognition and killing—are a third class of recognition strategy. We reasoned that extensible AND gate circuits could be built by combining multiple orthogonal synNotch receptor platforms. To construct a three-input AND gate, we could use our Gal4- and LexA-based synNotch platforms to recognize the first and second priming antigens and a CAR to

recognize the third antigen. These receptor components can be linked in different configurations, akin to electronic circuits: in series (where synNotch A induces expression of synNotch B, which in turn induces expression of CAR C), or in parallel (where synNotch A induces expression of part 1 and synNotch B induces expression of part 2 of a split CAR) (Fig. 3, A and B). We engineered both such configurations, using a first synNotch receptor that recognizes epidermal growth factor receptor (EGFR) and a second that recognizes MET. For the in-series circuit, the dual synNotch cascade drove expression of an anti-HER2 CAR (fig. S7A). For the in-parallel circuit, the same two synNotch receptors drove two different parts of a split anti-HER2 CAR [in this case, part 1 was a secreted CAR adapter that had an anti-HER2 scFv linked to a peptide neo-epitope (PNE) tag (22), and part 2 was a CAR that recognizes the PNE tag] (fig. S9A).

The in-series three-input cascade circuit showed precise recognition in multiple in vitro assays, including cell killing and proliferation (Fig. 3, C to E, and fig. S8C). These T cell outputs were only observed when presented with target cells expressing all three of the required antigens. In contrast, the in-parallel circuit was imperfect, showing partial killing of one of the two antigen combinations (EGFR⁺/HER2⁺) (fig. S9B). This is likely because of small amounts of leaky expression by the anti-MET synNotch, such that when the partner (anti-EGFR) synNotch is stimulated, some amount of the complete anti-HER2 split CAR is generated, resulting in killing activity. The linkage of these same synNotch receptors in series, which is predicted to lead to multiplicative specificity, provides an effective mechanism to suppress the impact of individual receptor leakiness (23, 24), although with slowed killing kinetics. We also identified other design principles for constructing efficient synNotch recognition cascades, focusing on the sequence of deploying the Gal4- and LexA-based receptors (Gal4 has stronger output than LexA) (fig. S7C). We built versions of the synNotch cascade with either order: Gal4→LexA (strong→weak) or LexA→Gal4 (weak→strong) (fig. S7D). We found that only the strong→weak cascade effectively propagated signal to yield detectable output in response to multiantigen stimulation (the importance of antigen density to these designs is discussed in the supplementary text section of the supplementary materials). Thus, these synthetic receptor cascades can be an effective way to integrate multiple inputs, subject to several key design constraints.

Given the precise in vitro function of this three-input AND gate in-series circuit, we tested whether T cells bearing this circuit could selectively kill tumors in vivo using mouse tumor models. We constructed target K562 tumor lines that were either MET⁺/EGFR⁺/HER2⁺ (target) or MET⁺/HER2⁺ (control) (fig. S8A) and implanted them in a bilateral tumor model in NSG mice (Fig. 3F). Both of these tumors contain the CAR killing antigen (HER2) and thus could be killed if the circuit was substantially leaky. The three-input AND gate T cells were administered by tail vein injection and allowed to autonomously explore and act on both tumors. We found that the T cells showed the same precise recognition observed in vitro—the three-input T cells were able to rapidly clear the three-antigen tumors, while ignoring the two-antigen tumors on the opposing flank (Fig. 3F; see fig. S10, B and C, for repeat experiment at different dosage). These results show an extensible strategy for building higher-input AND gate recognition circuits.

A fourth recognition strategy that we explored involves building circuits with flexibility of recognition. Although increasing selectivity of recognition is generally desirable, intrinsic heterogeneity or loss of particular antigens could require increased flexibility of recognition in particular dimensions. Recent studies have shown that tandem CARs—CARs with two different antigen recognition heads—provide a mechanism for engineering OR gates that can kill on the basis of either of two possible antigens (25-29). Such OR-killing functionality could be achieved by having a synNotch receptor induce expression of a tandem CAR. We tested whether we could also build tandem-headed OR gate synNotch receptors and incorporate them into higher-order circuits to accommodate priming antigen-specific recognition flexibility (Fig. 4A). A tandem synNotch receptor targeting EGFR and HER2 functions well to induce reporter expression in response to either individual target antigen (Fig. 4B). We incorporated this tandem synNotch into a three-antigen circuit (tandem synNotch→MET CAR), yielding T cells that robustly kill target cells that express MET combined with either EGFR or HER2 (Fig. 4C). Thus, it is possible to build OR gates into either synNotch or CAR receptors, and synNotch→CAR AND gates can flexibly recognize both priming and/or killing antigens. This gives control of how tightly or loosely a region of antigen-space is targeted to optimize effective recognition (Fig. 4D and fig. S13).

Together, these results show the power of cellular engineering as opposed to molecular engineering (21, 30, 31)—by engineering cellular regulation, we can integrate multiple molecular recognition events to achieve more precise and robust recognition and discrimination of target cells (e.g., cancer cells). Components such as synNotch do not carry out a prespecified physiological output function but rather execute the generic function of inducing transcriptional expression. Thus, they function as connectors that allow different molecular recognition events and outputs to be linked in different sequential configurations. By combining molecular recognition events with intracellular regulatory circuits, a diverse array of selective recognition programs can be engineered. Here we show that a sensor cell (for example, a therapeutic T cell) can be flexibly engineered to recognize combinations of intracellular and extracellular antigens and to integrate information from one, two, or three antigens using AND/OR/NOT logic (Fig. 4E). These modular and hierarchically designed cell circuits are compatible with new target antigens and new kinds of molecular recognition platforms that may emerge.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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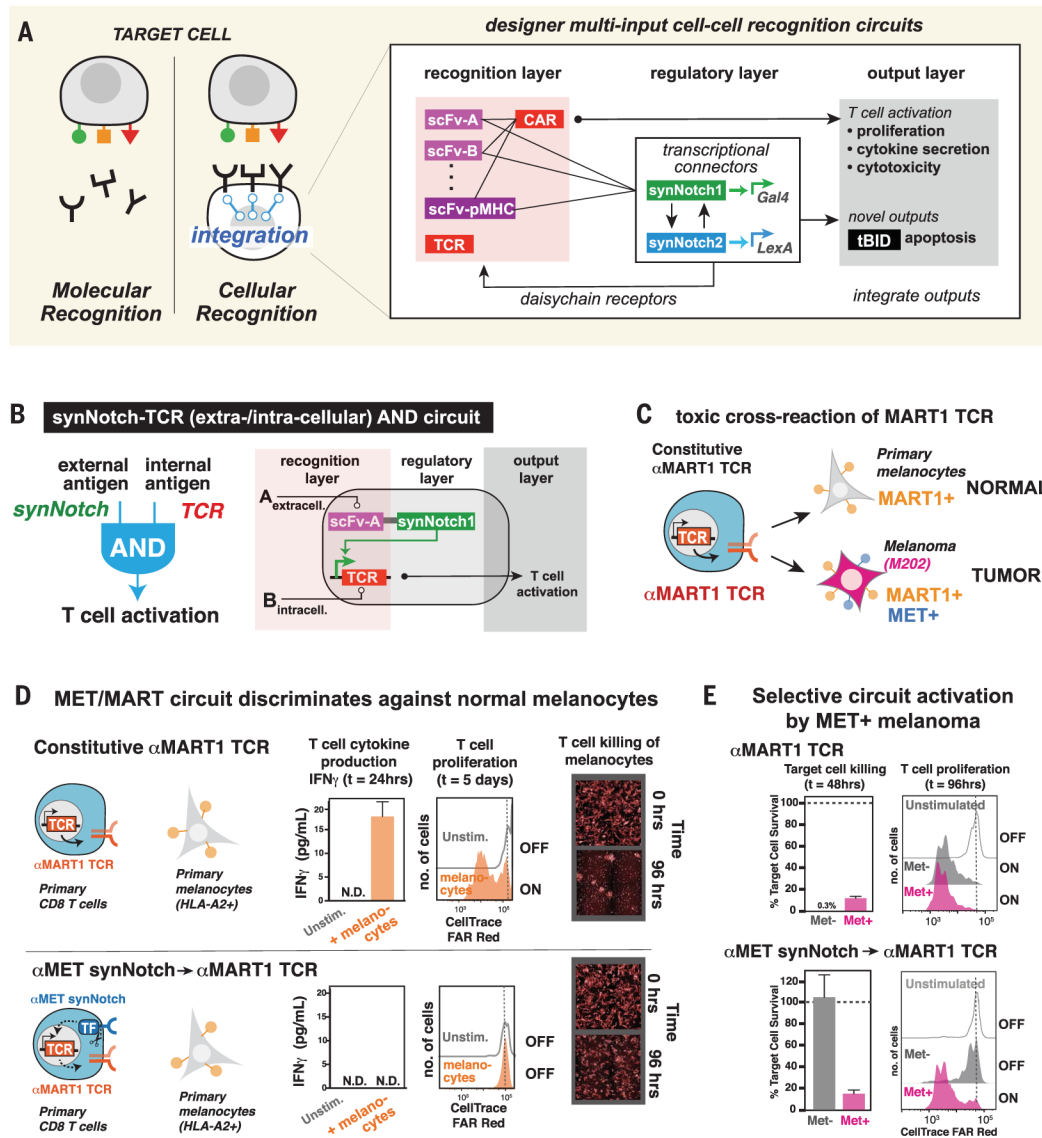


Fig. 1. SynNotch modules can be used to daisy-chain multiple receptor systems to design diverse multi-input cell-cell recognition circuits, including combinatorial recognition of internal and external antigens.

(A) Recognition of disease cells currently focuses on individual molecular recognition events (e.g., antibodies and CARs). In principle, cell therapies can be engineered to integrate multiple molecular recognition events into a composite cellular recognition program. Transcriptional connectors, such as synNotch receptors, provide a modular way to functionally link multiple receptors. Here the recognition layers are composed of scFvs or similar antigen binding molecules used by CAR or synNotch receptors. SynNotch receptors act as a regulatory layer inducing antigen-triggered expression of synthetic transcription factors (in this case, GAL4 or LEXA) that control cell function within the output layer. (B) Intracellular and extracellular antigen information could be integrated into a T cell recognition program by using a synNotch receptor that drives expression of a TCR. (C) MART1 TCR is example of TCR that requires additional inputs to eliminate toxic cross-

reactions—the TCR targets both melanomas and normal melanocytes (MART1 is also referred to as MLANA). We find that a subpopulation of patient-derived melanoma cells also express additional tumor-associated antigens, such as MET (fig. S1A). **(D)** Experimental validation that anti-MET synNotch→anti-MART1 TCR circuit improves discrimination of anti-MART1 TCR against normal melanocytes. Primary human CD8 T cells were engineered with a constitutive anti-MART1 TCR or an anti-MET Gal4-VP64 synNotch→anti-MART1 TCR circuit. These T cells were cocultured at 1:1 effector: target cell ratio (E:T) for varying times with primary human melanocytes that were HLA-A2⁺/MART1⁺/MET⁻. The supernatant from these cocultures was collected at 24 hours and analyzed for interferon- γ (IFN- γ) levels via an enzyme-linked immunosorbent assay. After 5 days of coculture, remaining cells were collected and T cell proliferation was determined by measuring CellTrace dye dilution via flow cytometry. synNotch→TCR circuit T cells showed no activation in response to the normal melanocytes (cytokine production: $n = 3$, error bars are SD; N.D., not detected; * $P < 0.01$; proliferation: representative of three independent experiments). In separate cell-killing experiments, melanocytes were labeled with CellTrace Far Red, and confocal microscopy images were taken after coculture with constitutive or synNotch-induced MART1 TCR T cells. Representative images show that constitutive anti-MART1 TCR T cells cleared the primary melanocytes, whereas neither untransduced nor synNotch→TCR circuit T cells showed any reactivity (see fig. S1E for larger images). **(E)** The anti-MET synNotch→anti-MART1 TCR circuit is still effective at recognizing and killing MET⁺MART1⁺ melanoma cells. T cells with constitutive or synNotch-induced MART1 TCR were cocultured with MET⁻ (M262) as well as the MET⁺ (M202) cell lines at an E:T of 1:1. Cytotoxicity was measured by flow cytometry as the number of tumor cells remaining compared with untransduced T cells after 48 hours, and proliferation was measured by dilution of CellTrace Far Red after 5 days. * $P < 0.01$. Representative of three independent experiments.

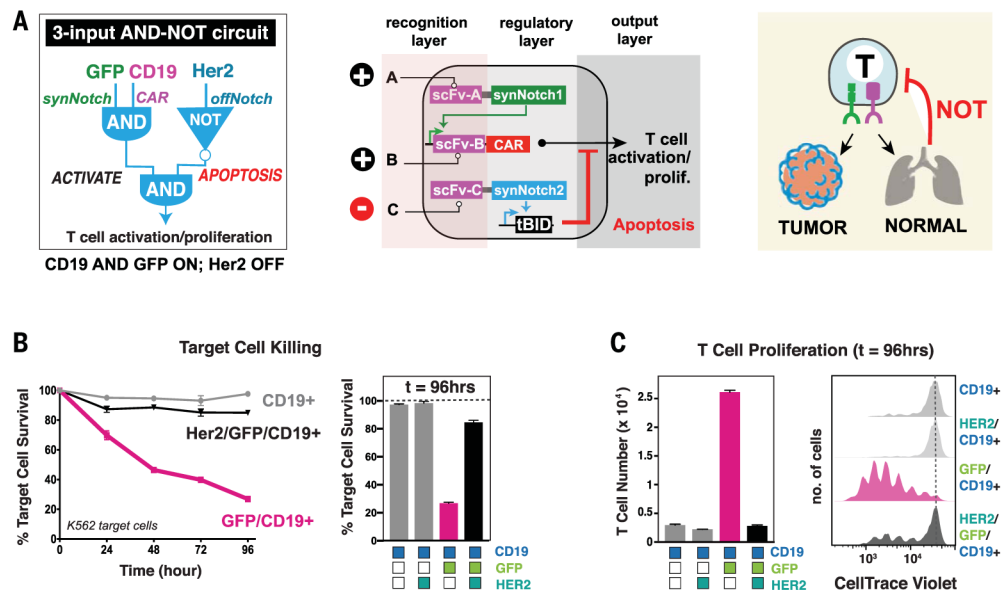


Fig. 2. Engineering recognition circuits that combine positive and negative logic by using synNotch modules that drive opposing outputs of activation and apoptosis.

(A) Design of a three-input circuit integrating an AND gate with a NOT gate to control T cell activation. The anti-GFP LEXA synNotch induces expression of the anti-CD19 CAR, and the orthogonal anti-HER2 GAL4 synNotch induces expression of proapoptosis protein tBID (“offNotch”). These T cells must first bind surface GFP to induce CAR expression and should only activate and kill target cells if the T cells sense both surface GFP and CD19. If the T cells sense HER2, then tBID induction will trigger apoptosis, which should deplete the effector T cell population and serve as an antigen-dependent NOT gate to prevent target cell killing. (B) Quantification of replicate target cell killing data gathered via flow cytometry after 1:1 E:T cocultures of varied times between engineered CD8 T cells with the circuit shown in (A) and K562 target cells expressing different combinations of CD19, surface GFP, and HER2. Although in the first 24 hours the T cells kill some of the HER2/GFP/CD19⁺ off-target cells, there is no further killing at later time points. In contrast, the T cells continue to kill the GFP/CD19⁺ on-target cells over the 96-hour time course ($n = 3$, error bars are SD), $*P < 0.01$ compared with CD19⁺ control. Representative of three independent experiments. (C) Histograms of T cells stained with CellTrace Violet dye in the coculture experiment described in (B) and processed via flow cytometry after 96 hours. After 96 hours, cell counts (left) and CellTrace plots (right) show proliferation of T cells only in the presence of GFP/CD19⁺ target cells and NOT HER2/GFP/CD19⁺ target cells (representative of at least three independent experiments) ($n = 3$, error bars are SD), $*P < 0.01$ compared with CD19⁺ control. See figs. S3 to S6 for more details on construction and analysis of this circuit and its components.

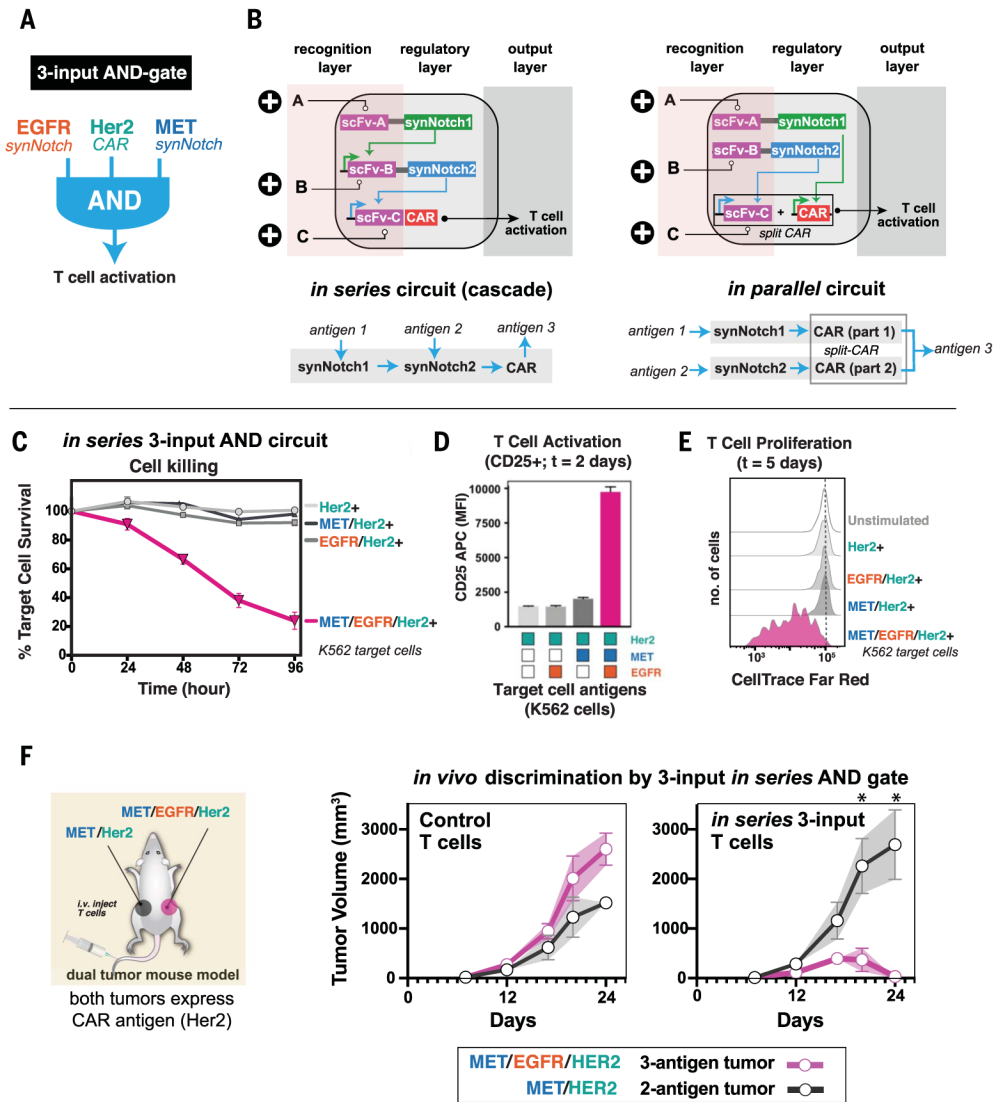


Fig. 3. Multi-synNotch circuits: Design and in vivo validation of ultraprecise three-input AND gates.

(A) Target design of a three-input AND gate circuit (must have EGFR, HER2, and MET for activation). (B) Two alternative design schemes for using synNotch and CAR components to build three-antigen AND gate recognition circuits. Both schemes use two synNotch receptors, but akin to an electrical circuit, the receptors can be functionally arranged to be in-series or in-parallel. The in-series three-input circuit has a first synNotch receptor (recognizes antigen 1) that, when activated, induces expression of a second synNotch receptor (recognizes antigen 2), which in turn, when activated, induces expression of a CAR (recognizes antigen 3, the killing antigen). In contrast, the in-parallel circuit uses the two different synNotch receptors (recognize antigens 1 and 2) to each induce components of a split CAR, which only when coexpressed will recognize antigen 3 (killing antigen). See figs. S7 to S9 for details on construction and testing of both of these circuits and their components. (C) Selective killing by CD8⁺ T cells transduced with the in-series three-input AND gate circuit. T cells with the full EGFR synNotch 1 \rightarrow MET synNotch 2 \rightarrow HER2 CAR

circuit only killed K562 cells expressing all three antigens (pink line). No killing was observed of target cells with two or fewer of the antigens. Killing was measured by flow cytometry every 24 hours and normalized to effect of UnT CD8⁺ T cells ($n = 3$, error bars are SD). Representative of three independent experiments. **(D)** Selective T cell activation, as measured by CD25 staining of T cells [as in (C)], was only seen when T cells were stimulated with K562 cells expressing all three antigens. **(E)** Selective proliferation of three-input T cells (using CellTrace Far Red–stained T cells) was only observed under stimulation with K562 cells expressing all three antigens, $*P < 0.01$ compared with HER2⁺ control. **(F)** In vivo validation of three-input T cells. These T cells selectively kill triple-antigen-positive tumors while sparing double-antigen-positive tumors in a bilateral tumor mouse model. 5e6 K562 tumor cells were implanted into the flanks of NSG mice (left flank: MET⁺/HER2⁺; right flank: MET⁺/HER2⁺/EGFR⁺). Seven days after tumor implantation, 3e6 control untransduced T cells or MET-AND-EGFR in-series CAR T cells were administered by tail vein injection. Complete clearance of triple-antigen-positive tumor was seen, while tumor lacking one of the priming antigens (EGFR) was spared ($n = 5$ mice, error bars are SEM, significance determined by Holm-Sidak t -test, $*P < 0.05$). Individual mouse plots and 6e6 T cell dose plotted in fig. S10.

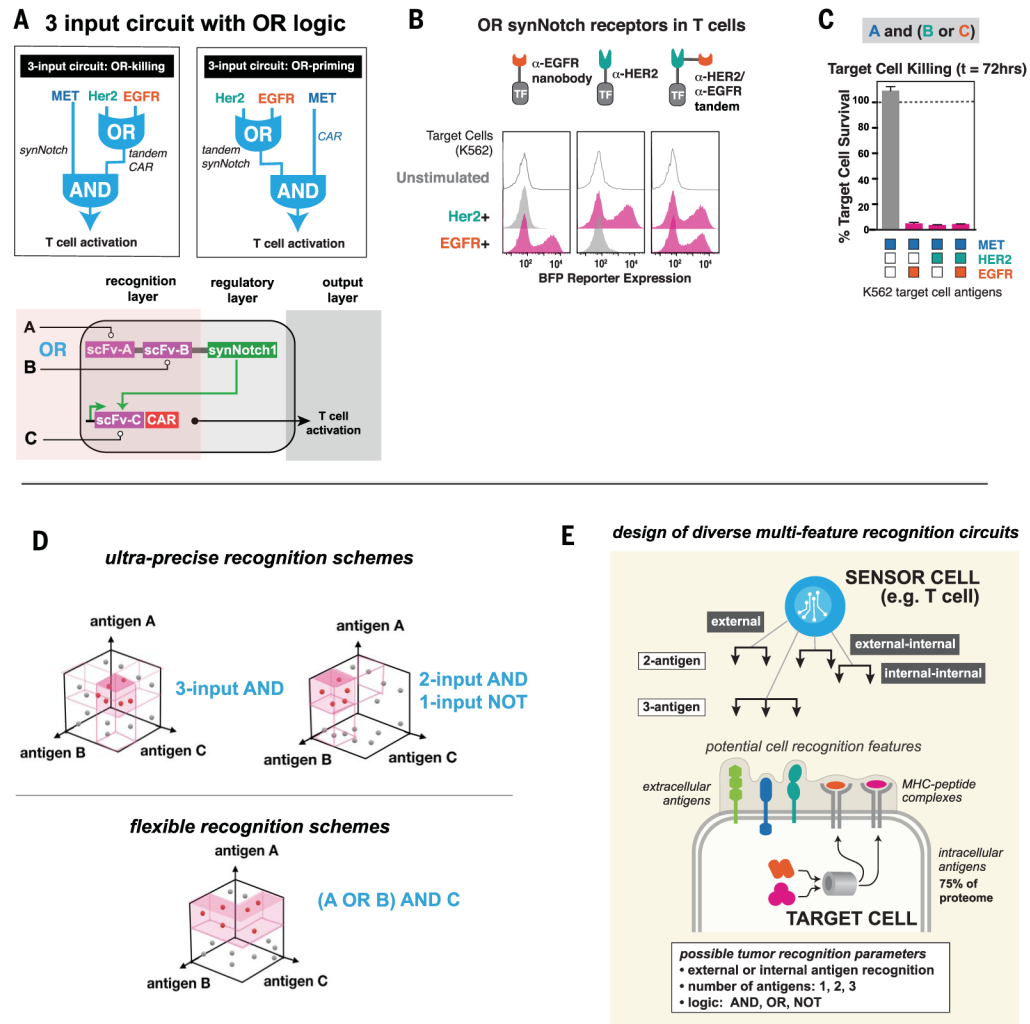


Fig. 4. Diverse cell-cell recognition schemes can be engineered using synNotch integrative circuits.

(A) Three-input circuits that incorporate AND and OR logic to introduce increased recognition flexibility of alternative antigens (in this case, recognition of [HER2 OR EGFR] AND MET). Alternative designs involve use of either tandem CARs or tandem synNotch receptors (each with two antigen recognition domains). Detailed scheme for constructing an OR-AND three-input circuit using a tandem synNotch receptor (see fig. S11 for more detail). (B) Validating OR recognition function of a tandem synNotch receptor that recognizes both EGFR and HER2. Primary human CD8 T cells were engineered with an anti-EGFR synNotch, anti-HER2 synNotch, or anti-HER2/EGFR tandem synNotch controlling the expression of a BFP reporter. All synNotch receptors used a Gal4-VP64 transcription factor. These T cells were cocultured at 1:1 E:T for 24 hours with K562 target cells expressing HER2, EGFR, or neither antigen as labeled. Histograms of BFP levels measured via flow cytometry show that the dual synNotch specifically responds to either HER2 OR EGFR positive target cells, whereas the two single-antigen synNotch receptors only respond to one of the target cells (representative of three independent experiments). (C) Efficient OR killing output by CD8 T cells transfected with the above circuit. T cells

efficiently kill K562 cells that express [MET and Her2] or [MET and EGFR], but not MET alone ($n = 3$, error bars are SD). **(D)** We demonstrate that three-antigen circuits can be engineered with different user-desired recognition behaviors—one can engineer ultraprecise A-and-B-and-C or A-and-B-not-C circuits, or more-flexible recognition schemes that tolerate heterogeneity in particular dimensions of antigen space [A-or-B]-and-C. It may now be possible to program therapeutic cells capable of recognizing any possible combinatorial antigen signature desired. **(E)** T cells can be engineered with an assortment of diverse combinatorial antigen recognition circuits. Information from up to three receptors targeting different antigen inputs can be integrated to generate highly specific recognition programs that integrate intracellular and extracellular targets as well as AND, NOT, and OR logic. Such recognition circuits should also function in other engineered cell types. See fig. S12 for a summary “periodic table” of general multiantigen cell-cell recognition circuits that are possible.